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Deposition of Selected Airborne Particles Into a Microfluidic Flow Cytometer for Bioanalysis

**by Hermes Huang, Yong-Le Pan,
Steven C. Hill, and Richard K. Chang**

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14. ABSTRACT In a proof-of-concept experiment, we demonstrate how selected airborne bacteria, detected in air by light scattering, can be deflected aerodynamically into the input well of a microfluidic flow cytometer and analyzed using fluorescein-labeled antibodies. This experiment demonstrates a new method for introducing airborne samples into a microfluidic cell. The results suggest that by deflecting only those airborne particles having the fluorescence spectra of bioaerosols and by depositing the individual bioaerosols into the smallest volume of reagent required for analysis, it should be possible to achieve a system that can continuously monitor the air for certain bioaerosols while minimizing reagent usage.					
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1. Introduction

1.1 The Problem: Efficient Collection of Aerosols for Rapid Continuous Analysis

There is a need for instruments that identify airborne infectious agents and other biological particles such as allergens and toxins. Airborne transmission of diseases to humans, other animals, and plants is common, and it occurs in places such as hospitals, schools, and agricultural facilities. For many monitoring applications it would be desirable to have instruments that can run continuously and provide a rapid response. Optical detection techniques, such as ultraviolet laser-induced fluorescence (UV-LIF) (Pan et al., 2003; Eversole et al., 2001), laser induced breakdown spectroscopy (Hybl et al., 2006), and mass spectrometry techniques (Murphy et al., 2003) provide a rapid single-particle response, but lack the specificity needed for species- or toxin-specific identification.

For identification of aerosols, biochemical assays such as an antibody (Stopa 2003) or nucleic acid assays (Easley et al., 2006) can provide high specificity (Hindson et al., 2005). However, these assays require aqueous solutions and expensive reagents, and often cannot be performed quickly in bulk solutions. Continuous operation of such assays would tend to require significant amounts of reagents, can be expensive and can increase the need for operator time. Microfluidic devices (e.g., McClain et al., 2001; Easley et al., 2006) help solve the reagent usage problem by drastically reducing the volume of reagents required to perform a bioassay and reducing the time required to perform an assay. However, the problem of efficiently introducing aerosol samples into microfluidic devices without wasting both collected samples and liquids remains as a major challenge, and is the main focus of this paper.

1.2 Microfluidic Analysis

There are large ongoing efforts to develop improved methods for analyzing microorganisms, mammalian cells, protein toxins (Kartalov et al., 2006), and other materials in microfluidic devices. On-chip detection of cells labeled with fluorescent dyes or fluorescent-tagged antibodies is described by McLain et al., (2001), Dittrich and Schwille (2003), and Sakamoto et al., (2005). An integrated microfluidic genetic analyzer, which accepts whole blood samples and does PCR analysis well enough to identify *B. anthracis* spores in less than 30 minutes in asymptomatic mice has been demonstrated (Easley et al., 2006). Reasons that microfluidic systems are replacing many wet-chemistry testing systems is that the volume of reagents required and waste generated can be tiny; temperatures can be controlled rapidly, and diffusion distances and times can be small. These characteristics lead to quicker and more efficient assays.

Whitesides (2006) in his review of microfluidic devices states, “Before . . . samples can be analyzed by microfluidic devices, they must be converted to a form that is compatible with the

intended analysis, and then introduced into the analytical device. The procedures required to complete these tasks are surprisingly sample-dependent, and not necessarily ‘micro’ in scale. He also mentions that the option to introduce the sample as a powder into the microfluidic device would be ideal. Our efforts in this report relate to introducing dilute, airborne powder samples efficiently into a microfluidic device.

1.3 Collection of Particles into Liquid for Analysis

Collecting particles from air and continuously injecting these particles into a microfluidic device with minimal loss of particles and liquid is not a trivial task, especially if one objective is to minimize total liquid usage.

Collecting particles onto a surface or a filter, and then eluting the particles into liquid by washing them from the surface or filter and injecting this liquid into a microfluidic device is one traditional approach. However, such an approach may be subject to problems of particles remaining attached to the surface or filter; these particles may not be analyzed, or they may become resuspended and contaminate later samples. Also, automating such systems for continuous operation and a rapid response may be problematical.

Samplers which collect airborne particles directly into liquid are typically inertia based. Examples are impingers (Crook 1995) and wet cyclones (Birenzvige et al., 1998). Some electrostatic based samplers, for example, the TSI Electrostatic Air Sampler (EAS, Model 3100), can collect particles into water (Mainelis et al., 1999).

None of the methods described above are specific for certain types of particles. Instead they collect all aerosols which fall within a certain size range. Samplers that do collect particles directly into liquid use volumes of liquid that are also many times larger than the volumes required for analysis in a microfluidic cell. If one were able to collect specific aerosol particles into a volume of liquid comparable with the typical sample volume of a microfluidic cell, one would be able to substantially increase the efficiency of microfluidic-based identifier.

1.4 Selection of Airborne Particles that have Specific Optical Characteristics

In our previous work (Pan et al., 2003; 2004; Davitt et al., 2006) we have shown that airborne particles can be selected based upon their UV-LIF spectra and deflected into an area of about 7 mm^2 without, and about 1 mm^2 with a particle aerodynamic localizer (Pan et al., 2004; Frain et al., 2006) using an air puffer. Thus, rather than collecting all the airborne particles present for analysis, we can selectively collect the potentially interesting particles. We have surveyed the ambient air in Adelphi, MD (Pinnick et al., 2004). It was found that about 11 % of ambient aerosol particles emit significant fluorescence, and can be grouped into 8 subcategories based on their UV-LIF spectra. This suggests that if one is only interested in bioaerosols falling within one of these subcategories, then by using UV-LIF as a triggering signal, one could reduce by at least an order of magnitude the number of particles the identification system would have to

examine. For example, out of the 11% of ambient aerosols with a significant UV-LIF signal, typically less than 1% of the aerosols at Adelphi, MD had a fluorescence spectrum similar to *B. subtilis*. This ability to select particles could be especially useful when trying to sample large volumes of air, because optical detection systems like our LIF detector are capable of measuring fluorescence spectra of many tens of thousands of particles per second (90,000 in our case). By making use of this high throughput through the LIF classifier and the ability to select specific individual aerosol particles based on its results, the load on a downstream identification system could be significantly reduced, even when the total number of particles screened in air could be very large. In the case of a microfluidic-based identifier, this could allow for reduced flow rates, reagent usage, and probability of clogging.

1.5 Objective: Collect Selected Particles Directly into Input Well of Microfluidic Analyzer

This report's objective is to describe a way of selectively deflecting airborne particles into a small volume of liquid for further analysis by biochemical means, specifically the liquid within the input well of a microfluidic cell. We conducted a proof-of-concept experiment where elastically scattered light provided a signal that a particle was present and the particle was deposited into the open input well of a microfluidic cell (figure 1). Fluorescein-labeled antibodies to *E. coli* present in the input well bind to any *E. coli* cells present in the airborne particles, and the cells are counted in the microfluidic flow cytometer based on immunofluorescence. Elastic light scattering instead of fluorescence is used in our proof-of-concept experiment because the system is easier to assemble, and allows us to focus on the problem of collection of puffed particles into an input well. For more selectivity in triggering the puffer, other rapid discrimination or classification methods such as UV-LIF can be used.

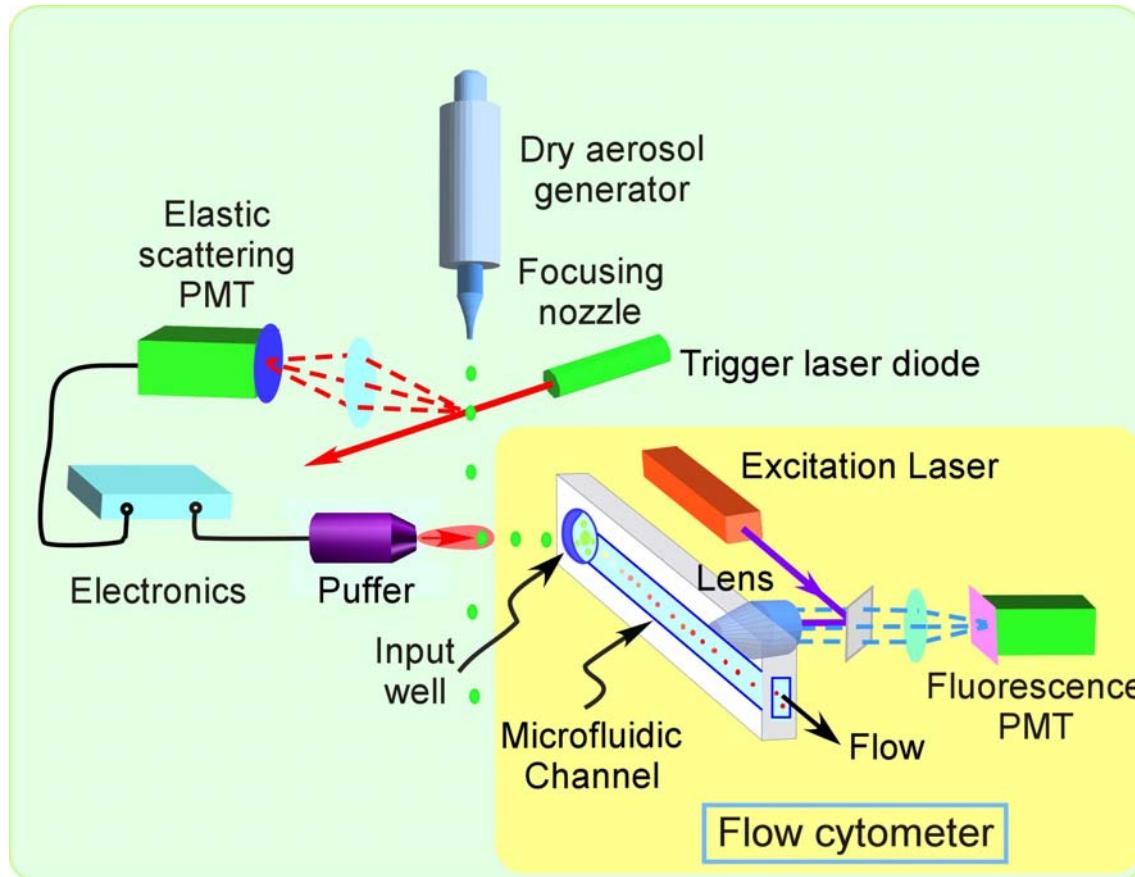


Figure 1. Schematic of setup for proof-of-concept experiment: Dry aerosol particles are generated and collimated via an inkjet aerosol generator. Light scattering detects when a particle flows past the trigger volume, which then triggers the puffer to deflect the particle into the microfluidic cell. Within the microfluidic cell fluorescein-labeled antibodies bind to bacteria within the aerosol particle, and fluorescence is counted via laser excitation and a photomultiplier tube.

The concept instrument that we describe and illustrate here can be thought of as the coupling of two flow cytometers. First, the air-flow cytometer (Shapiro, 2003, page 10) classifies particles in air according to an optical signal. The puffer deflects the particles according to that signal. The deflected particles move on a trajectory such that they impinge on the liquid in the sample-input well of the second cytometer. Then, fluorescein-labeled antibodies in the input well bind to particles that have the appropriate antigens, and these fluorescein-labeled particles are counted in the liquid-flow cytometer. The puffer serves as the bridging technology between the two flow cytometers which are working in different media (first air, then liquid).

This experiment demonstrates a key technology required to achieve a continuous rapid-response identification system for bioaerosols while minimizing reagent usage. The experiment suggests the possibility of a continuously running, low-reagent-usage identifier for airborne bioparticles which: selects the particles in air and deposits them directly into the input well of a microfluidic single-particle analyzer, and consequently collects and analyzes only those airborne particles that

have laser-induced fluorescence consistent with bioparticles of interest, so that clogging and reagent usage can be minimized.

2. Experimental Methods

2.1 Detection, Collection, and Analysis Apparatus

The detection collection and analysis apparatus, as illustrated in figure 1, consists of two main parts: (1) a trigger and puffer system, and (2) a microfluidic flow cytometer. Also illustrated in figure 1 is the ink-jet aerosol generator used for testing the setup.

2.1.1 Trigger and Puffer System

Rather than using a fluorescence-cued triggering system (Pan et al., 2004), we chose to use a much simpler light scattering-based trigger. The use of elastic light scattering reduces the complexity of the overall system and allows us to focus on how effectively the puffer can deposit airborne particles into solution within an open microfluidic well. Any triggering system can be used as long as it is capable of determining rapidly (before the aerosol has travelled more than a few millimeters) whether or not a particle is of interest.

The light scattering-based triggering system consists of a 670 nm diode laser focused onto a spot in the particle stream, a lens to collect the scattered light, and a photomultiplier tube to measure the scattered light. Whenever a particle passes through the trigger beam, the transient pulse of scattered light at a specific angle Θ and Φ is detected by the photomultiplier, which then triggers the puffer.

The puffer used in this experiment is a new version of our air-based deflector used in our previous bioaerosol detection system (Pan et al., 2004). It is a solenoid-based valve from which a very small burst of compressed nitrogen is used to redirect particles in the air into the open input well of the microfluidic flow cytometer in response to a trigger signal from the photomultiplier tube.

2.1.2 Microfluidic Flow Cytometer

An Agilent cell fluorescence LabChip was used as the microfluidic cell for our flow cytometer in this experiment. This LabChip is typically used with the Agilent Bioanalyzer 2100 system. The channels have a cross section of $75 \mu\text{m} \times 25 \mu\text{m}$. The buffer flow limits the sample flow to about half of the channel width.

A custom-made epifluorescence microscope was set up around the LabChip for fluorescence excitation and detection, as illustrated schematically in figure 1. In order to see significant

contrast when a bacterium, 1-2 μm in diameter and tagged with fluorescent-labeled antibodies, passes by in the presence of unbound fluorescent-labeled antibodies, the laser and detector were focused using a 60x long-working-distance microscope objective (0.70 numerical aperture) into a cylinder of about 20 μm in diameter on one side of the channel. This relatively small diameter region reduces the contribution to the background fluorescence from the unbound antibodies. If a bacterium passes through the channel outside of this detection region, it cannot be detected.

The 496 nm line of an argon-ion laser was used for the fluorescence. A CCD camera was used for alignment and imaging, while a photomultiplier tube was used for the fluorescence measurements. An oscilloscope provided trigger pulses when the photomultiplier output exceeded threshold conditions, and a TTL counter kept track of the number of fluorescence events detected.

For the purposes of this experiment, only one fluid channel of the six available channels on the Agilent LabChip was used. All unused channels were sealed. The buffer flow was fed with a phosphate-buffered saline solution, and the sample input well was initially filled with a solution containing fluorescent-labeled antibodies to the bacteria (here, *E. coli*) in the test particles. A syringe pump was used to apply a constant vacuum flow at the waste outlet of the Labchip. At the flow rate used, the antibody solution was consumed at a rate of about 75 $\mu\text{L}/\text{hr}$, which if run continuously would result in less than 2 ml/day being consumed.

2.2 Test Bacteria and Antibodies

Heat-killed, lyophylized *E. coli*, O157:H7 (KPL, Gaithersburg, MD) was used as the test bioaerosol sample. Fluorescein-labeled antibodies targeted at this strain of *E. coli* were also obtained from KPL. The concentration given in the datasheet ($3 \times 10^9/\text{mL}$) is used to calculate the concentrations in all solutions made.

2.2.1 Bacterial Aerosol Generation

An inkjet-aerosol generator (IJAG), made by Edgewood Aerosol Science, was used to produce liquid droplets about 50 μm in diameter. These droplets were air dried by passing them through a heated column to form dry aerosol particles. A nozzle at the end of the drying column then collimated the particles to form an aerosol stream (of less than 1 mm diameter).

Typically, a solution of *E. coli* with a concentration of 9×10^{11} cells/L was placed into the cartridge of the IJAG and aerosolized, and so within each 50- μm droplet, there were on average about 59 *E. coli* cells. Thus, each particle deflected was assumed to contain about 59 *E. coli* cells as well as non-bacterial material in the cell suspension that had been lyophylized.

2.2.2 Antibodies and No-Wash Fluorescent Antibody Assay for *E. coli*

Fluorescein-labeled antibodies to *E. coli*, O157:H7 were obtained from KPL (Gaithersburg, MD). Because we desired to illustrate our concept for single-aerosol-particle analysis, sorting, and deposition into a microfluidic flow cytometer in the simplest manner that is adequate, we used an assay in which bacteria are directly deposited into a solution that contains fluorescent-labeled antibodies that are specific to the bacteria of interest. No wash steps were used to remove the unbound labeled antibodies (discussed in section 4).

2.3 Validation and Alignment of the Microfluidic Flow Cytometer

Once the epifluorescence microscope was set up, fluorescent polystyrene microspheres suspended in water were deposited into the input well via a micropipette. Microspheres ranging in size from 1 μm to 8 μm were used, and all could be easily detected visually using the CCD camera. The signal level seen by the photomultiplier tube was very high compared with the background noise, with a signal-to-noise ratio typically above 10.

Before each test using the puffer, the alignment of the optics was checked by running 10 μL of a dilute fluorescein solution through the microfluidic channel, which allowed the sample flow to be visualized under laser illumination with all of the optical filters in place. Then 40 μL of DI water was run through the system to clean out the fluorescein solution before the antibody solution was introduced into the microfluidic cell. The antibody solution was run for at least 5 minutes to establish the background fluorescence level before any *E. coli* was introduced, either via puffer or micropipette.

3. Results

3.1 Use of No-Wash Bacterial Assay in LabChip

Because our goal is a proof-of-concept experiment for a continuously running identifier, and because we are unaware of any commercially available microfluidic flow cytometer that performs a wash step to remove unbound antibodies, we first demonstrated that we were able to detect *E. coli* in the LabChip against the background fluorescence of the unbound fluorescent-labeled antibodies.

E. coli was mixed with the dilute antibody solution, allowed to incubate for 5 minutes, and then introduced into the microfluidic cell. Signals similar to those from 2- μm fluorescent-doped polystyrene microspheres were observed that were above the background fluorescence, which was now dominated by the unbound fluorescent-labelled antibodies. The signal-to-noise ratio was lower than that seen with pure fluorescent polystyrene microspheres, because of the

fluorescence from unbound antibodies. Example signals detected with the PMT are shown in figure 2.

When *B. subtilis* was mixed with the same antibody solution, no significant fluorescence signals could be seen above the background. Finally, a mixture of both *B. subtilis* and *E. coli* was added to the antibody solution, and only certain particles fluoresced when crossing the region illuminated by the laser (a backlight was used so that all particles could be seen optically).

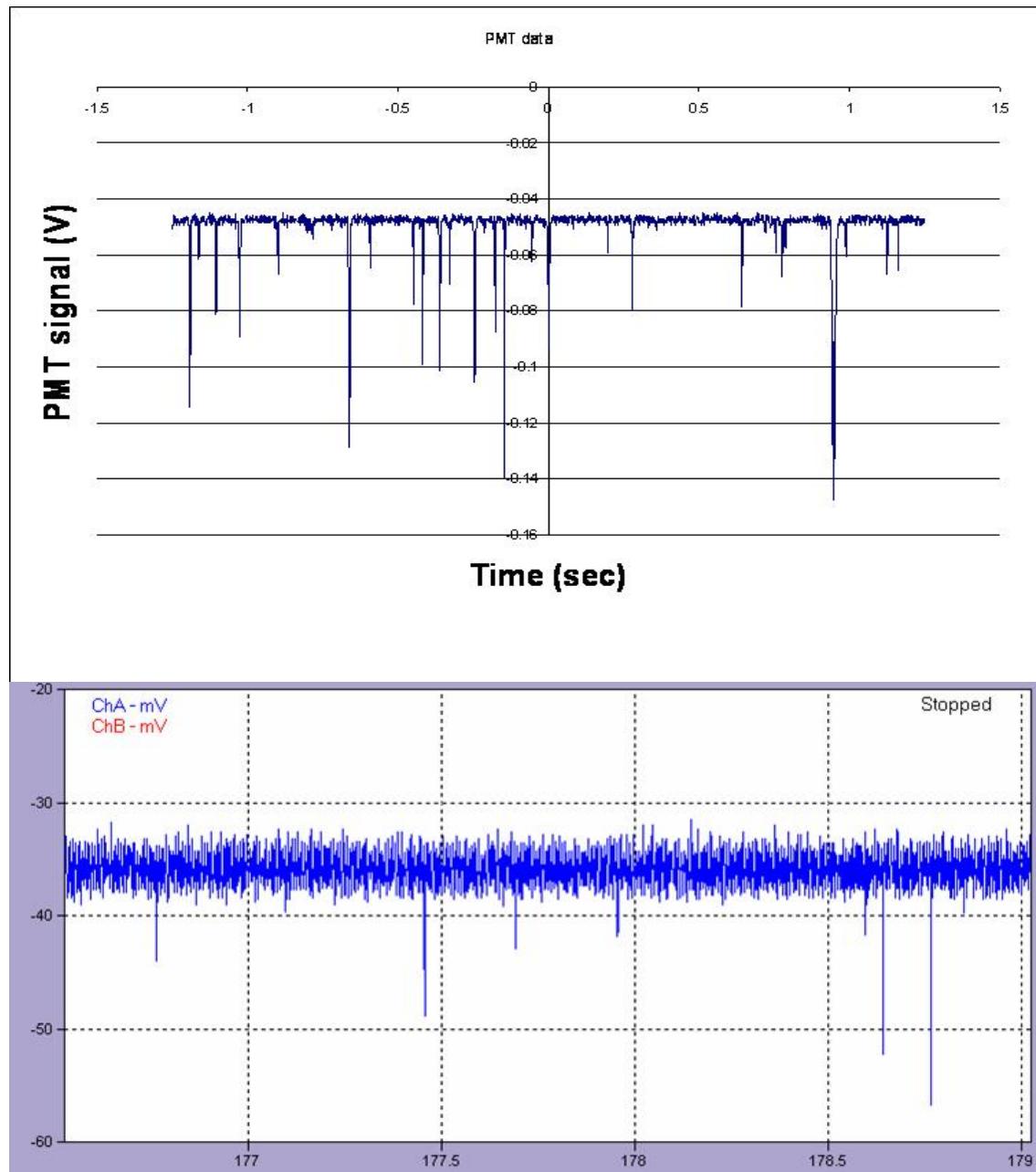


Figure 2. (a) Photomultiplier signal from 2 μ m fluorescent polystyrene microspheres. (b) Photomultiplier signal from *E. coli* in a solution of fluorescent antibodies.

3.2 Efficiency of Detection of Labeled *E. coli* Placed Directly in the LabChip

To determine the detection efficiency of the microfluidic flow cytometer with our setup and *E. coli*, we mixed a solution of *E. coli* with the solution that contained the fluorescent-labeled antibodies. Then the resulting solution, containing about 3390 *E. coli* cells, was deposited directly into the input well using a micropipette. The fluorescence events were counted, with all other conditions kept the same as when aerosolized bacteria were analyzed. When the fluid well had completely emptied, 263 fluorescence events were counted, which is an efficiency of 7.75%.

Sakamoto et al., (2005) have done experiments where *E. coli* cells were counted using the cell fluorescence Labchip within the Bioanalyzer 2100 system, and their calibration curves using fluorescent microspheres shows that even within the Bioanalyzer 2100 system, one only sees fluorescence events for approximately 5% of all fluorescent particles present. Thus, we feel our custom setup compares favourably with the Bioanalyzer 2100 system for which the Labchip was designed.

3.3 Collection and Analysis of Puffed Particles in the Labchip

In our experiments we typically filled the input well with 40 μL of fluorescent-labeled antibody solution, so that the fluid level is slightly above the level of the rim in the input well. We then aerosolized *E. coli* cells using the methods described in section 2.1. In our experiments, typically each particle generated is a clump of approximately 59 cells. Typically some set number of particles were puffed and then the particle generator and puffer were turned off, typically within less than 5 minutes. The liquid level has not changed significantly during this time because the flow rate into the cytometer is small. Usually, within minutes of the first puffer activity, fluorescence events were seen in the channel of the flow cytometer. We counted fluorescence events until the sample well was emptied, which took approximately 35 minutes. The control experiment described in section 3.2 is then performed immediately afterwards by pipetting the solution directly into the microfluidic well without moving anything, and with no cleaning step in between.

We measured the percentage of puffed bacteria that were detected in the flow cytometer. Then, the ratio of this efficiency of detection in the LabChip of puffed bacteria to the efficiency of detection of bacteria in the LabChip (from section 3.2), is the collection efficiency, or percentage of puffed particles that were collected in the input well. Since the control experiment is done immediately afterwards, the conditions are as similar as possible, and if any *E. coli* remain in the well this would only overestimate the efficiency calculated in section 3.2 so that the overall collection efficiency would be underestimated.

We found that the collection efficiency depends sensitively on several parameters, such as position of puffer relative to the light-scattering detectors, timing of the pulse to the puffer, puffer nozzle alignment and distance from well, and pressure of gas. The sensitivity of the collection efficiency to these parameters, and the need to reposition various components in order

to refill the input well, etc., resulted in more variation in collection efficiency than might otherwise be obtained if fewer realignments were required, as would be the case if we had been able to obtain a microfluidic flow cytometer designed for continuous input of liquid into the input well. Here we describe in detail one specific case and calculate the overall efficiency and collection efficiency for this case.

To estimate the collection efficiency, we aerosolized *E. coli* cells using the methods described in section 2.1 where each particle generated is a clump of approximately 59 cells. After 50 scattering events (2950 *E. coli* cells, based on the calculated average), the IJAG was turned off. A total of 62 fluorescence events were counted by the time the microfluidic well emptied, to give a 2.1% efficiency. This is a combined collection-detection efficiency.

The control experiment described in section 3.2 was performed immediately following the experiment, yielding a 7.75% efficiency. Using these two efficiencies we estimate that the collection efficiency for puffing the bacteria into the input well is about 2.1 / 7.75, or 27%. That is, 27% of the puffed particles were deposited into the input well.

4. Discussion

We have demonstrated that bacteria-containing particles can be selected rapidly in air according to a single-particle optical signal (in our demonstration, light scattering intensity), and then deposited into a microfluidic bioanalyzer (in our demonstration a flow cytometer), and then analyzed using biorecognition molecules (in our demonstration, fluorescent-labeled antibodies).

4.1 No-Wash Assay

An antibody assay without a wash step avoids both the time and complexity an off-chip wash step would entail. This is important for the design of a rapid bioaerosol identifier, since an off-chip wash step would increase cost and response time. Stopa (2000) has shown that a fluorescent antibody assay (with no wash step) for *Bacillus anthracis* spores in a flow cytometer has a detection limit of approximately one organism per microliter. While other assays, such as sandwich assays, or nucleic-acid based assays typically have even higher specificity for a target, Stopa's results suggests that a no-wash antibody assay may be adequate for some purposes, especially if more than one antibody is used for each analyte in the assay.

Because of advances in on-chip cell- and bead-manipulation technology, the approach of using no off-chip wash step does not mean that no wash steps can be used. For example, even in a system no more complex than the LabChip we used, because the fluorescent labelled antibodies diffuse faster than the bacteria, once the sample flow meets the buffer flow, the concentration of fluorescent-tagged antibodies near the cells should begin to decline. Microfluidic devices can be designed to optimize this diffusion away from the cells before the fluorescence is measured.

Alternatively, dielectrophoresis (Lagally et al., 2005) may be used to pull the cells from the antibody-laden flow into a cleaner buffer flow before measurement.

4.2 Detection Efficiency

The goal of the experiment was not to find the optimal assay for identification of bioaerosols, but to demonstrate a method to perform an in-solution, biochemical assay on particles that are initially dry and airborne, and where this method can be rapid and can require little reagent usage. Thus, we paired a commercially available microfluidic chip with a commercially available fluorescent-antibody assay. If one wishes to develop a bioaerosol identifier, one would optimize both the microfluidic cell and the assay to match the target bioaerosol and the expected environment. Such a microfluidic system would probably incorporate features which were not on the Labchip we used, such as a continuous feed system with a feedback loop which maintains a constant liquid level at the input well. Such a continuous feed system could strongly reduce the need to realign the optics every time additional liquid was added to the input well. The flow channels would be designed to be the optimal size for the size of particles one would like to detect, and methods such as those mentioned in section 4.1 for separating unbound antibodies may be incorporated to increase the signal-to-noise ratio.

4.3 Collection Efficiency

We stress in this experiment that we are demonstrating our ability to collect select particles into a microfluidic cell and perform an antibody assay on them. Since this experiment represents a proof-of-concept rather than a thorough exploration of how parameters such as pulse width, airspeed, and gap distance affect deposition efficiency into solution, we expect to be able to increase the collection efficiency by optimizing these conditions.

We have also shown that for deposition onto a surface, a regrouper, which is like a funnel that refocuses the puff of air carrying the selected particle, can increase the collection efficiency into a small area (Frain et al., 2006). A properly designed system which employs a regrouper should increase the collection efficiency of particles into the liquid in an input well of a microfluidic analyzer. However, designing and assembling a system that uses the regrouper well, where the end of the regrouper nozzle is close to the liquid surface, will require time and funds, and probably only makes sense to do when it is combined with a microfluidic analyzer that is designed for a continuous feed of liquid into the input well. All of these optimizations are very doable but not trivial or inexpensive.

5. Conclusion

This experiment demonstrates the concept of using an air-flow cytometer to detect and select particles in air according to some optical property, and to deflect certain of these particles directly into the sample-input well of a microfluidic flow cytometer. This technique of aerodynamically deflecting specific particles into an input well of a microfluidic cell is a promising technology for low-reagent-usage bioaerosol analyzers. Incorporation of a UV-LIF-spectrum-based discriminator rather than the elastic scattering signal used in the demonstration here would provide a way to select for further analysis in the microfluidic device only aerosols with LIF spectra similar to particles of interest. The types of assay which can be performed and the types of aerosol which can be identified are only limited by the capabilities of microfluidic analysis systems.

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